Should Lignin be Redefined?

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Lignin is that stuff that pulp and paper industries work hard to remove from wood in order to produce paper. It binds up wall carbohydrates in forages so they have limited digestibility, and too much of it in certain vegetables results in a poor quality food that is tough and woody in texture. Is there nothing good that we can say about this molecule? Of course, it is important in terms of adding structural strength to wood for construction; and there is evidence that lignin may be important in human nutrition for aiding digestion, lowering cholesterol, and perhaps reducing the risk of colon cancer. Perhaps it is a bit narrow to think of lignin as just some negative factor that limits our exploitation of plants.

Just what is this material called lignin that is deposited in virtually every cell wall of every vascular land plant? There are two ways to define lignin— from a chemical point of view (i.e., its chemical composition and structure) or from a functional view that stresses what lignin does within the plant. As for the chemical definition, it has been recognized for 50 years now that lignin is a polymeric material composed of phenylpropanoid units from three simple compounds (monolignols): *p*-coumaryl, coniferyl and sinapyl alcohol (Fig. 1). Until recently it was believed that only these phenolics were involved in the synthesis of lignin.

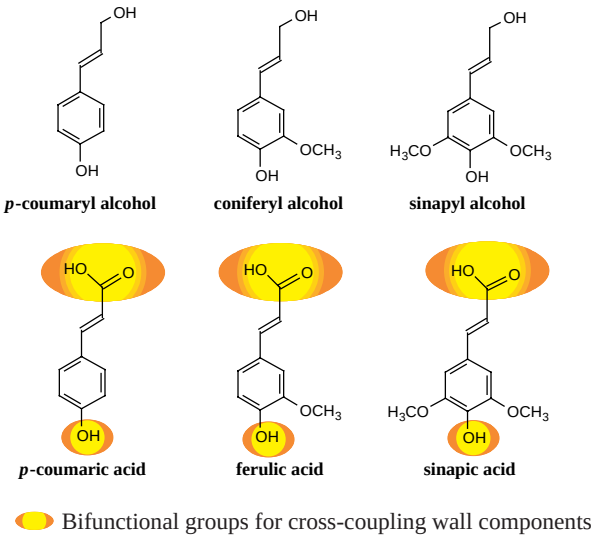


Figure 1. Major products of the phenylpropanoid pathway that are incorporated into plant cell walls.

Structurally, lignin is a polymer built from the more or less random coupling of the three monolignol units (Fig. 1) into the growing polymer.

From a functional point of view, lignin imparts strength to wall matrices, has a critical function in terms of water transport, and impedes the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects and other herbivores. As we have investigated the structural nature of lignin, we are understanding more about what it is doing within the plant. The functional role of lignin has not changed, but our strict compositional definition is no longer accurate. So what has changed?

As new analytical techniques have been applied to the study of lignin, evidence is rapidly mounting that forces us to critically evaluate and ultimately reject a number of accepted dogma. Lignin is not about a polymer simply composed of three monolignols!! It is a much more dynamic structure that is formed from the elegant incorporation of key wall phenolics to produce a functional molecule. The key, as far as the plant is concerned, is producing a functional polymer, but not necessarily restricting its synthesis to the three recognized monolignols. There have been hints of the dynamic nature of lignin for some time now; we have erroneously chosen the most simplistic view. Perhaps we simply wanted to deny any additional complexity to a molecule that already is impossible to investigate as a complete entity.

Let us examine the cross-linking story as an example. Cross-linking of lignin to wall polysaccharides has been speculated upon for 20 to 30 years, but the definitive proof always seemed to be just beyond reach. The discovery of hydroxycinnamates in walls (particularly of grasses) led to the speculation that they might be involved in cross-linking due to their bifunctional nature (Fig.1). As it turns out, *p*-coumarates are quite prominent yet appear to have no crosslinking role. Ferulates, on the other hand, have been shown to be covalently linked to lignin. It is interesting to note that even though ferulates are quite

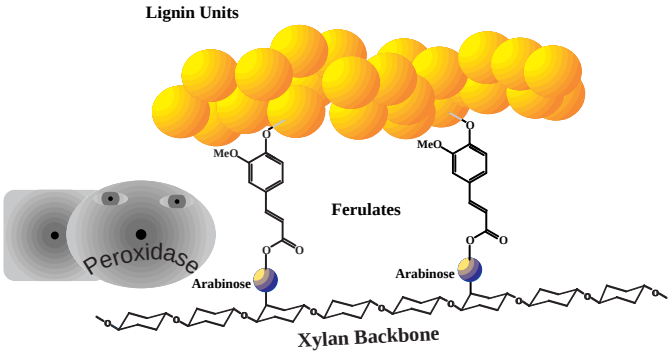


Figure 2. Schematic representation of ferulate attachment to lignin via simple ether linkages.

similar in structure to coniferyl alcohol, which incorporates nicely into lignin structures with complex non-repeating covalent bonds, it was generally believed that ferulic acid attachment was restricted to a simple ether linkage (Fig. 2). Such attachment would render ferulates completely hydrolyzable by high temperature alkaline treatment. One could picture it as ferulates attached only to the outer surfaces of the lignin molecule (whatever an outer surface would be, see Fig. 2). This assumes that the preferred reaction would be the addition of the phenolic hydroxyl to a quinone methide (an opportunistic reaction).

Although this is a nice picture that makes us feel good, we now know that it does not represent the reality of ferulate interactions with lignin or its involvement in the lignification process. Ferulates undergo extensive coupling with lignin particularly during the initial stages of lignin formation. The radical coupling that takes place parallels that of coniferyl alcohol and therefore is so extensive (both in the sense of bond types and numbers) that much of the wall-bound ferulate is no longer extractable from the lignin molecule (Fig. 3). So

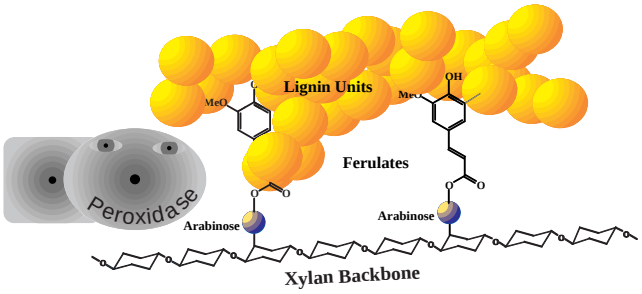
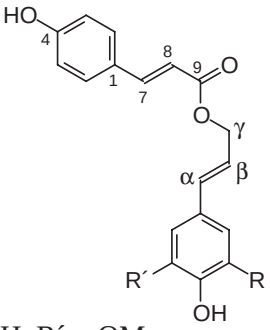


Figure 3. Schematic representation of ferulate incorporation into lignin polymers via several types of covalent linkages.

now we have a "lignin" that contains the normal monolignols but also contains this other molecule intimately incorporated into the growing polymer. Is this still lignin?

What of the *p*-coumarates? It has been recognized for some time now that *p*CA is covalently linked to lignin subunits. These molecules do indeed "hang off" the lignin structure by a simple ester bond. Early assumptions tended to take the most simplistic view, i.e., free *p*CA within the wall matrix would add to growing lignin polymers through quinone methide intermediates to form a-ester linkages. However, structural analysis some 30 years ago suggested a major portion of the *p*CA was on the g-carbon, not the a-carbon (Shimada et al., 1971). This has recently been confirmed and indeed shown that all *p*CA within detectable limits is attached to the g-carbon. Since there is no radical mechanism that would give rise to this attachment, it must be formed via enzyme activity. There are two possibilities. The first is that there is an apoplastic enzyme that can attach *p*CA to accessible Cg-OH groups after the lignin polymer is formed. Plausible, but for what function? The second possibility is a cytoplasmic enzyme that couples *p*CA to coniferyl or sinapyl alcohol (Cg-OH) forming *p*-coumarate conjugates (Fig. 4) that are transported to the wall matrix as a unit. This seems the more likely possibility in light of recent evidence indicating a potential role of hydroxycinnamates in aiding the formation of sinapyl alcohol-rich lignins. (Takahaama et al. 1996). In the latter case the plant is forming a unique molecule (sinapyl *p*-coumarate) that deviates from the "normal" lignin monolignols but is incorporated into lignin.



Coniferyl alcohol R = H, R´ = OMe
Sinapyl alcohol R = R´ = OMe

Figure 4. Ester linkage of p-coumarate acid to monolignols.

As yet another example, let us consider the maize brown midrib mutant bmr_3. It is known that this mutant contains a lower syringl/guaiacyl ratio due to reduced activity of an *O*-methyl transferase (Grand et al. 1985) involved in sinapyl alcohol formation. Lapierre et al. (1988) demonstrated that bmr_3 incorporates 5-hydroxyconiferyl alcohol (an intermediate in the formation of sinapyl alcohol, see Fig. 1) into its lignin. The mutation leading to reduced *O*-methyl transferase activity may not result in reduced lignin content but it does alter its composition.

In a similar fashion a pine mutant that has severely reduced activity of CAD (cinnamyl alcohol dehydrogenase) incorporated coniferylaldehyde into its lignin. The CAD enzyme reduces coniferylaldehyde to coniferyl alcohol as the last step of monolignol biosynthesis. With this enzyme blocked, the pine incorporates the intermediate into its lignin. The pine is also unique in that it was not a simple tradeoff of coniferylaldehyde for coniferyl alcohol in the lignin; there were also large amounts of dihydroconiferyl alcohol indicating a significant divergence from the current monolignol biosynthetic pathway (Fig. 5). The impact of these changes on the properties of lignin are not fully understood at this time, nor is their impact upon wall cross-linking or, ultimately, on wall degradation. What it does indicate is a significant latitude for lignin structure. The plant is utilizing

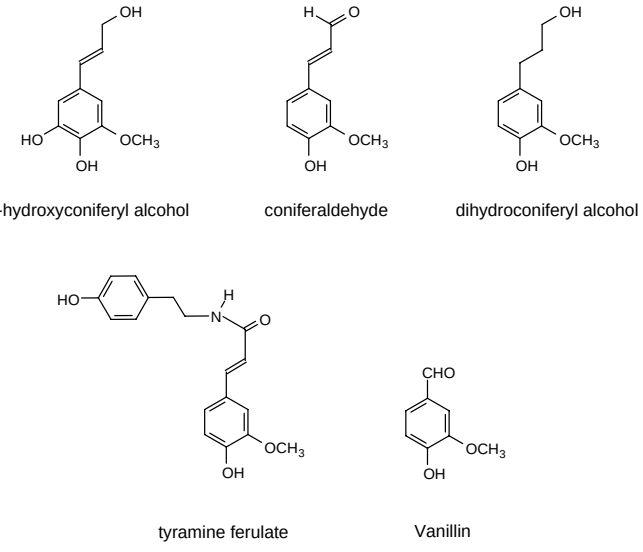


Figure 5. Non-traditional phenolics that are incorporated into lignin isolated from natural mutants and molecularly altered plants.

available molecules that polymerize into lignin efficiently even though they are not the "normal" lignin building blocks.

Attempts to alter lignin concentration in plants has focused on the genes encoding the various enzymes within the monolignol biosynthetic pathway (Boudet and Grima-Pettenati 1996; Boudet et al. 1995; Whetten and Sederoff 1995). The last two enzymes of the lignin pathway (cinnamoyl CoA reductase, CCR and cinnamyl alcohol dehydrogenase, CAD) are good candidates for genetic manipulation of lignin in that they are solely involved in monolignol biosynthesis. One, therefore, does not have to be concerned about altering some other metabolic pathway that may be crucial to plant viability (e.g., flavonoid or phytoalexin pathways). Efforts to alter lignin through the down-regulation of CAD have been successful to some degree (Baucher et al. 1996; Halpin et al. 1994; Higuchi et al. 1994). In all cases, there was a definite shift in the lignin composition towards higher levels of cinnamaldehyde units and lower coniferyl alcohol units. However, total lignin levels were virtually unchanged though there was a change in the alkaline solubility of lignin. The CCR altered plants did show reduced lignin levels (50% !!), although the plants were not as physically robust. Recent ^{13}C-^{1}H NMR work analyzing ^{13}C-enriched lignins isolated from tobacco lines with down regulated CAD (by antisense genes) and other tobacco lines with down regulated CCR (by antisense genes) revealed the incorporation of further non-traditional components into their lignins (Fig. 5; Ralph et al. 1998). The antisense CAD tobacco contained lower coniferyl alcohol and higher levels of cinnamaldehydes along with elevated levels of benzaldehydes with little change in Klason lignin levels. The results from genetically manipulated plants and from the natural mutants (bmr and pine CAD deficient) clearly indicate the metabolic plasticity involved in the lignification process. There are many unanswered questions as to the impact of these alterations on wall degradation, but it indicates the wide potential for altering lignin structure.

It is also clear that lignin can no longer be thought of as a molecule composed solely of varying ratios of coniferyl, sinapyl, and *p*-coumaryl alcohols. Like it or

not, plants produce functional lignins that are mildly or extensively contaminated with other components. These components are inextricably bound and cannot be separated from the "pure lignin" polymers because they have been intimately incorporated into the lignin structure by the radical coupling reactions that define lignification. As complex as lignin was before, it now seems structurally even more complex. However, this complexity is entirely logical from a mechanistic point of view and the metabolic plasticity may be evolutionarily wise.

Lignin — THINK DIFFERENTLY!

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